

## Gene Family with Transformation Modulating Activity

The work leading to this invention was supported in part by Grant No. RO1 CA 54404 from the National Institutes of Health. The U.S. Government retains certain rights in this invention.

### BACKGROUND

#### File of the Invention

This invention is directed to various members of a gene family with transformation 10 modulating activity, and to diagnostic and gene therapy techniques based on the variants.

#### Review of Related Art

Prostatic adenocarcinoma is the most frequent malignancy in adult men with approximately 317,000 new cases diagnosed each year (Parker, et al., CA, 46:8-27, 1996). In spite of the capabilities for early diagnosis and treatment (Potosky, et al., JAMA, 15 273:548-552, 1995), it represents the second leading cause of cancer death in men following lung cancer.

To date, the study of alterations in specific genes has not been particularly rewarding in primary prostate cancer. Most alterations in the widely studied oncogenes and tumor suppressor genes occur in only 20 - 30% of primary prostate carcinomas, except 20 for the myc gene, where overexpression has been observed in as many as 50 - 60% of such cases (Fleming, et al., Cancer Res., 46:1535-1538, 1986). Up to 40% of primary prostate cancers studied by comparative genomic hybridization display chromosomal aberrations (Visakorpi, et al., Cancer Res., 55:342-347, 1995), although such alterations occur more frequently as tumors recur and become refractory to hormonal therapy. Characterization 25 of candidate proto-oncogenes or tumor suppressor genes at such altered loci may eventually shed light on tumor progression in the prostate.

pp32 (GenBank HSU73477) is a highly conserved nuclear phosphoprotein. Increased expression of pp32 or closely related species is a frequent feature of clinical cancers. For example, in human prostate cancer, high-level expression of RNA 30 hybridizing with pp32 probes occurs in nearly 90% of clinically significant prostate cancers, in contrast to the substantially lower frequencies of alterations of other oncogenes

and tumor suppressors (See U.S. Patent No. 5,726,018, incorporated herein by reference).

Molecular Features and Activities of pp32.

pp32 is a nuclear phosphoprotein that is differentiation-regulated during differentiation of adult prostatic epithelium (Walensky, et al., Cancer Res. 53:4720-4726, 1993). The human pp32 cDNA sequence (Gen-Bank U73477) is 1052 bp in length and encodes a protein of 249 amino acids. The protein is composed of two domains: an amino terminal amphipathic  $\alpha$ -helical region containing a leucine zipper, and a highly acidic carboxyl terminal region. The murine and human forms of pp32 are highly conserved with over 90% nucleic acid homology and over 95% protein-level homology.

Human pp32 has been isolated independently by a number of groups. Vaesen et al. ("Purification and characterization of two putative HLA class II associated proteins: PHAPI and PHAPII." *Biol. Chem. Hoppe-Seyler*, 375:113-126, 1994) cloned an essentially equivalent molecule, termed PHAPI, from an EBV-transformed human B-lymphoblastoid cell line; PHAPII, cloned by the same strategy, is unrelated to pp32. This study identified PHAPI through its association in solution with human HLA class II protein, noting membrane and cytoplasmic localization as well as nuclear; the gene has putatively been localized to chromosome 15q22.3-q23 by fluorescent in situ hybridization (Fink, et al., "Localization of the gene encoding the putative human HLA class II-associated protein (PHAPI) to chromosome 15q22.3-q23 by fluorescence in situ hybridization." *Genomics*, 29:309-310, 1995). More recently, a group studying inhibitors of protein phosphatases identified pp32 as I1PP2a, an inhibitor of protein phosphatase 2a (Li, et al., "Molecular Identification of I1 PP2A, a novel potent heat-stable inhibitor protein of protein phosphatase 2A." *Biochemistry* 35:6998-7002, 1996); another phosphatase inhibitor, I2PP2a, is unrelated to pp32. Interestingly, another recent report (Ulitzur, et al., "Biochemical characterization of mapmodulin, a protein that binds microtubule-associated proteins." *Journal of Biological Chemistry* 272:30577-30582, 1997) identified pp32 as a cytoskeletally-associated cytosolic protein in CHO cells. It is not clear whether this finding stems from a difference in system, or whether pp32 can localize to the cytoplasm under certain circumstances. pp32 has also been identified as LANP, a leucine rich nuclear protein in the central nervous system (Matsuoka, et al., "A nuclear factor containing the

leucine-rich repeats expressed in murine cerebellar neurons. *Proc Natl Acad Sci USA* 91:9670-9674, 1994).

There are also a number of reports of gene products bearing lesser degrees of homology to pp32. The Vaescn group has identified a series of unpublished sequences, termed PHAPI2a (EMBL Locus HSPHAPI2A) and PHAPI2b (EMBL Locus HSPHAPI2B), also cloned from an EBV-transformed human B-lymphoblastoid cell line. These variant pp32 sequences are distinct from the sequences reported herein, representing the April protein instead. April, cloned from human pancreas, is shorter than PHAPI2a by two N-terminal amino acids (Mencinger, et al., "Expression analysis and chromosomal mapping of a novel human gene, APRIL, encoding an acidic protein rich in leucines." *Biochimica et Biophysica Acta*. 1395:176-180, 1998. see EMBL Locus HSAPRIL); PHAPI2b is identical to a subset of APRIL. Silver-stainable protein SSP29 (unpublished GenBank Locus HSU70439) was cloned from HeLa cells and is identical to PHAPI2a.

The nuclear phosphoprotein pp32 has been linked to proliferation. Malek and associates reported that various neoplastic cell lines showed markedly elevated expression levels and that bacterial polysaccharide induced expression of pp32 epitopes by B lymphocytes upon polyclonal expansion (Malek, et al., *J. Biol. Chem.*, 265:13400-13409, 1990). Walensky and associates reported that levels of pp32 expression, measured by *in situ* hybridization, increased in direct relation to increasing Gleason grade of human prostatic cancers.

pp32 cDNA probes hybridize strongly with prostatic adenocarcinoma, whereas the hybridization signal in normal prostate is confined to basal cells. Polyclonal anti-pp32 antibodies react strongly with sections of human prostatic adenocarcinoma. The antibodies and riboprobes used by the investigators in previous studies are consistent with cross-reactivities of the reagents with all reported members of the pp32 nuclear phosphoprotein family, therefore, while previous descriptions focused upon pp32, it cannot be excluded that homologous proteins were detected.

## SUMMARY OF THE INVENTION

In one aspect, this invention provides a DNA molecule containing at least a portion of the sequence consisting of base pairs 4894-4942 of the sequence shown in Figure 2 or

its complement. Alternatively, the DNA molecule may contain at least a portion consisting of base pairs 4879-4927, or base pairs 4858-4927. Alternatively, this invention provides a DNA molecule that contains at least a portion of a nucleotide sequence encoding amino acid residues 146-163 of tumor-derived pp32r1 sequence; preferably the DNA encodes all of that segment. In one mode, the DNA molecule is an expression vector which expresses said amino acid sequence, and the invention also includes a recombinant cell containing the expression vector. In another mode, the DNA molecule has the particular sequence operatively linked to a promoter in antisense orientation. In another alternative, this invention provides a DNA probe which specifically hybridizes on Northern blot with nucleic acid encoding the amino acids from residue 146-163 of the tumor-derived pp32r1 sequence, a preferred probe would have a sequence of at least 8 contiguous nucleotides "unique" to the nucleotide sequence of the pp32r1 variant as described herein. In yet another alternative, the invention provides a pair of nucleic acid primers each of which comprises at least 10 contiguous nucleotides, at least one of the primers binding specifically to the pp32r1 sequence, where if the primers are used in nucleic acid amplification of a suitable source of human nucleic acid, the amplification will produce an amplified nucleic acid encoding at least residues 146-163 of the pp32r1 sequence.

In still another aspect, this invention provides antibodies that specifically bind the tumor derived pp32, but do not bind to normal pp32. Preferably, these antibodies are monoclonal antibodies. The invention also provides polypeptides containing epitopes that bind these antibodies.

In yet another aspect, this invention provides diagnostic methods for predicting malignant potential of neuroendocrine, neural, mesenchymal, lymphoid, epithelial or germ cell derived tumors by determining, in a sample of human neuroendocrine, neural, mesenchymal, lymphoid, epithelial or germ cell derived tissue, the level of, or the intracellular sites of expression of, a gene product expressed from a gene sequence which encodes, *inter alia*, residues 146-163 of tumor derived pp32r1. Where the gene product is mRNA, the mRNA is extracted from the sample and quantitated, optionally by PCR, or the level of mRNA may be determined by *in situ* hybridization to a section of the tissue sample. Where the gene product is protein, the determination may include reacting the

sample with an antibody that specifically binds to tumor derived pp32, but not to normal pp32. Preferably, the tissue sample is carcinoma tissue, e.g., carcinoma or sarcoma of a tissue selected from the group consisting of epithelial, lymphoid, hematopoietic, mesenchymal, central nervous system and peripheral nervous system tissues, including colon carcinoma, prostate carcinoma and non-Hodgkin's lymphoma.

In still another aspect, this invention provides an androgen-activated transcriptional promoter which may be inserted into recombinant DNA molecules. The minimal promoter is made up of a transcription initiation site and at least one binding site for a steroid hormone receptor protein. Typically the consensus sequence for the steroid hormone receptor protein binding site is positioned within 5000 nucleotide base pairs (bp), more preferably within 3000 bp, or even fewer bp of the transcription initiation site. In a preferred mode, a number of binding sites for steroid hormone receptor proteins are positioned within that distance of the transcription initiation site, the promoter may contain five, ten or even 25 steroid hormone receptor protein binding sites. Preferably, the binding site(s) for steroid hormone receptor protein binding are selected from the consensus sequences listed on Table 1. In a preferred mode of the invention, the androgen-activated transcriptional promoter is operatively linked to an open reading frame comprising at least one exon of a protein coding sequence, operative linking of the open reading frame thereby providing an expression vector in which expression of the open reading frame is regulated by steroids.

In another aspect, this invention provides a method for screening candidate compounds for pharmacological activity by (1) culturing a cell transfected with the DNA molecule containing the androgen-activated transcriptional promoter which is operatively linked to an open reading frame comprising at least one exon of a protein coding sequence, and (2) determining expression of the open reading frame in the presence and absence of the compound. In a preferred mode the androgen-activated promoter may be all or an operative portion of the sequence in Figure 2 which is up-stream of the translation initiation site, or alternatively the androgen-activated promoter may be the 2700 bp of the sequence in Figure 2 which is upstream from the translation initiation site.

pp32 is a member of a highly conserved family of differentiation-regulated nuclear proteins that is highly expressed in nearly all human prostatic adenocarcinomas of Gleason Grade  $\geq 5$ . This contrasts with the low percentage of prostate tumors that express molecular alterations in proto-oncogenes or demonstrate tumor suppressor mutation or loss of heterozygosity. By analysis of specimens of human prostatic adenocarcinoma and paired adjacent normal prostate from three individual patients, the inventors have shown that normal prostate continues to express normal pp32, whereas three of three sets of RT-PCR-amplified transcripts from prostatic adenocarcinomas display multiple *cancer-associated* coding sequence changes. The cancer-associated sequence changes appear to be functionally significant. Normal pp32 exerts antineoplastic effects through suppression of transformation. In contrast, cancer-associated pp32 variants augment, rather than inhibit, transformation.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows detection of pp32-related mRNA in benign prostate and prostate cancer tissue sections by *in situ* hybridization.

Figure 1B shows immunohistochemical stain of prostate cancer sections with anti-pp32 antibodies.

Figure 2 shows the genomic sequence of variant pp32r1 isolated from human placenta.

Figure 3 provides a base-by-base comparison of the sequence of pp32r1 (top) with normal human pp32 (bottom). The numbering system for pp32r1 corresponds to Figure 1, and the numbering system for normal pp32 is taken from Chen, et al. Nucleotide base differences are underlined in the pp32r1 sequence. Sequences within the normal pp32 sequence missing in pp32r1 are represented by dashes. The open reading frame for pp32r1 is indicated by overlining.

Figure 4 shows the alignment of the pp32r1 amino acid sequence (top) with normal human pp32 (bottom). Residue changes are underlined in the pp32r1 sequence. Amino acids missing in the pp32r1 sequence compared to normal pp32 are represented by dashes.

Figure 5 shows the genomic sequence of variant pp32r2.

Figure 6A shows RT-PCR amplification of pp32 and pp32 variants from human prostate cancer and prostate cancer cell line.

Figure 6B shows cleavase fragment length polymorphism analysis of pp32 detects variant pp32 transcripts in human prostate cancer.

5        Figure 7 shows the alignment of nucleic acid (A) and amino acid (B) sequences from human prostatic adenocarcinoma and prostatic adenocarcinoma cell lines with pp32.

Figure 8 is a bar graph showing ras + myc induced transformed focus formation. Co-transfection with a pp32 expression vector reduces transformation, while co-transfection with a pp32r1 expression vector stimulates transformation.

10        Figure 9 is a bar graph showing pp32r1 stimulation of ras + myc induced transformed focus formation. Co-transfection with a pp32 expression vector reduces transformation, while co-transfection with expression vectors for pp32r1 sequences from prostate cancer cell lines stimulate transformation.

Figure 10 is a graph of transformation assay results for cells transfected with variant pp32 species, which are shown to stimulate transformation with variable potency.

#### 15        **DETAILED DESCRIPTION OF THE INVENTION**

The inventors have discovered that phenotypic changes in pp32 are a common feature of human prostate cancer. Previous data show that 87% of prostate cancers of Gleason Score 5 and above express pp32 or closely-related transcripts (U.S. Patent No. 20        5,734,022, incorporated herein by reference). This is striking in comparison to the frequency of molecular alterations in other widely studied oncogenes and tumor suppressor genes in primary prostatic adenocarcinoma, which occur in a substantially smaller proportion of cases. For example, myc overexpression (Fleming, et al.) occurs in around 60% of cases, and p53 is abnormal in only around 25% of primary tumors (Isaacs, et al., 25        in <sup>A</sup>Genetic Alterations in Prostate Cancer." Cold Spring Harbor Symposia on Quantitative Biology, 59:653-659, 1994).

Several lines of evidence suggest that pp32 may act as a tumor suppressor. Functionally, pp32 inhibits transformation *in vitro* by oncogene pairs such as ras with myc, mutant p53, Ela, or jun, or human papilloma virus E6 and E7 (Chen, et al., "Structure of 30        pp32, an acidic nuclear protein which inhibits oncogene-induced formation of transformed

foci." *Molecular Biology of the Cell*. 7:2045-2056. 1996). pp32 also inhibits growth of transformed cells in soft agar (Chen, et al.). In another system, ras-transfected NIH3T3 cells previously transfected to overexpress normal human pp32 do not form foci *in vitro* or, preliminarily, do not form tumors in nude mice, unlike control cells. In contrast, 5 knockout of endogenous pp32 in the same system by an antisense pp32 expression construct markedly augments tumorigenesis (Example 12 below).

In clinical prostate cancer, the situation at first appears counterintuitive. Most human prostate cancers seem to express high levels of pp32 by *in situ* hybridization (see Example 1 below) and stain intensely with anti-pp32 antibodies. Because pp32 inhibits 10 oncogene-mediated transformation (Chen, et al.), its paradoxical expression in cancer was investigated at the sequence level. The paradoxical question of why prostate cancers seem to express high-levels of an anti-oncogenic protein was addressed by comparing the sequence and function of pp32 species from paired normal prostate and adjacent prostatic carcinoma from three patients as well as from four prostate cancer cell lines. It is 15 demonstrated herein that pp32 is a member of a closely-related gene family, and that alternate expression of these closely-related genes located on different chromosomes modulates oncogenic potential in human prostate cancer. The variant pp32 species expressed in prostate cancer are closely related to pp32.

The present data indicate that prostate cancers express variant pp32 transcripts, 20 whereas adjacent normal prostate expresses normal pp32. Two instances clearly show that expression of alternate genes on different chromosomes can lead to the phenotypic switch, rather than mutation or alternate splicing. This switch in molecular phenotype is accompanied by a switch in functional pp32 phenotype. Normal pp32 is anti-oncogenic in character, in contrast to the pro-oncogenic variant transcripts that foster oncogene- 25 mediated transformation. The high frequency of this abnormality suggests that expression of variant pp32 species may play an etiologic role in the development of human prostate cancer. In addition, these findings have significant diagnostic and prognostic implications.



## Definitions

In describing the present invention, the following terminology is used in accordance with the definitions set out below.

### *Nucleic Acids*

5 In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

A DNA sequence "corresponds" to an amino acid sequence if translation of the  
10 DNA sequence in accordance with the genetic code yields the amino acid sequence (i.e., the DNA sequence "encodes" the amino acid sequence); one DNA sequence "corresponds" to another DNA sequence if the two sequences encode the same amino acid sequence.

Two DNA sequences are "substantially similar" when at least about 90% (preferably at least about 94%, and most preferably at least about 96%) of the nucleotides  
15 match over the defined length of the DNA sequences. Sequences that are substantially similar can be identified by the assay procedures described below or by isolating and sequencing the DNA molecules. See e.g., Maniatis et al., *infra*, DNA Cloning, vols. 1 and II *infra*; Nucleic Acid Hybridization, *infra*.

A "heterologous" region or domain of a DNA construct is an identifiable segment  
20 of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous region is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the  
25 genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A "coding sequence" or "open reading frame" is an in-frame sequence of codons that (in view of the genetic code) correspond to or encode a protein or peptide sequence.  
30 Two coding sequences correspond to each other if the sequences or their complementary

sequences encode the same amino acid sequences. A coding sequence in association with appropriate regulatory sequences may be transcribed and translated into a polypeptide *in vivo*. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence. A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. Promoter sequences typically contain additional sites for binding of regulatory molecules (e.g., transcription factors) which affect the transcription of the coding sequence. A coding sequence is "under the control" of the promoter sequence or "operatively linked" to the promoter when RNA polymerase binds the promoter sequence in a cell and transcribes the coding sequence into mRNA, which is then in turn translated into the protein encoded by the coding sequence.

Vectors are used to introduce a foreign substance, such as DNA, RNA or protein, into an organism. Typical vectors include recombinant viruses (for DNA) and liposomes (for protein). A "DNA vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. An "expression vector" is a DNA vector which contains regulatory sequences which will direct protein synthesis by an appropriate host cell. This usually means a promoter to bind RNA polymerase and initiate transcription of mRNA, as well as ribosome binding sites and initiation signals to direct translation of the mRNA into a polypeptide. Incorporation of a DNA sequence into an expression vector at the proper site and in correct reading frame, followed by transformation of an appropriate host cell by the vector, enables the production of a protein encoded by said DNA sequence.

An expression vector may alternatively contain an antisense sequence, where a small DNA fragment, corresponding to all or part of an mRNA sequence, is inserted in opposite orientation into the vector after a promoter. As a result, the inserted DNA will be transcribed to produce an RNA which is complementary to and capable of binding or hybridizing with the mRNA. Upon binding to the mRNA, translation of the mRNA is prevented, and consequently the protein coded for by the mRNA is not produced. Production and use of antisense expression vectors is described in more detail in U.S. Patent 5,107,065 (describing and exemplifying antisense regulation of genes in plants) and

U.S. Patent 5,190,931 (describing antisense regulation of genes in both prokaryotes and eukaryotes and exemplifying prokaryotes), both of which are incorporated herein by reference.

5 "Amplification" of nucleic acid sequences is the *in vitro* production of multiple copies of a particular nucleic acid sequence. The amplified sequence is usually in the form of DNA. A variety of techniques for carrying out such amplification are described in a review article by Van Brunt (1990, *Bio/Technol.*, 8(4):291-294). Polymerase chain reaction or PCR is a prototype of nucleic acid amplification, and use of PCR herein should be considered exemplary of other suitable amplification techniques.

#### 10 **Polypeptides**

For the purposes of defining the present invention, two proteins are homologous if 80% of the amino acids in their respective amino acid sequences are the same; for proteins of differing length, the sequences will be at least 80% identical over the sequence which is in common (i.e., the length of the shorter protein).

15 Two amino acid sequences are "substantially similar" when at least about 87% of the amino acids match over the defined length of the amino acid sequences, preferably a match of at least about 89%, more preferably a match of at least about 95%. Typically, two amino acid sequences which are similar will differ by only conservative substitutions.

"Conservative amino acid substitutions" are the substitution of one amino acid  
20 residue in a sequence by another residue of similar properties, such that the secondary and tertiary structure of the resultant peptides are substantially the same. Conservative amino acid substitutions occur when an amino acid has substantially the same charge or hydrophobicity as the amino acid for which it is substituted and the substitution has no significant effect on the local conformation of the protein. Amino acid pairs which may  
25 be conservatively substituted for one another are well-known to those of ordinary skill in the art.

The polypeptides of this invention encompass pp32r1 and pp32r1 analogs, pp32r2 and pp32r2 analogs, along with other variants of pp32 and their analogs. pp32r1 and pp32r2 are naturally occurring, mature proteins, and further encompass all precursors and  
30 allelic variations of pp32r1 and pp32r2, as well as including forms of heterogeneous

molecular weight that may result from inconsistent processing *in vivo*. An example of the pp32r1 sequence is shown in Figure 3, top line. "pp32r1 analogs" are a class of peptides which includes:

1) "Allelic variations of pp32r1," which are polypeptides which are substantially similar to pp32r1. Preferably the amino acid sequence of the allelic variation is encoded by a nucleic acid sequence that differs from the sequence of pp32r1 by one nucleotide in 300;

2) "Truncated pp32r1 peptides," which include fragments of either pp32 or allelic variations of pp32r1 that preferably retain either (i) an amino acid sequence unique to pp32r1, (ii) an epitope unique to pp32r1 or (iii) pp32r1 activity;

3) "pp32r1 fusion proteins," which include heterologous polypeptides which are made up of one of the above polypeptides (pp32r1, allelic variations of pp32r1 or truncated pp32r1 peptides) fused to any heterologous amino acid sequence.

"Unique" sequences of the pp32r1 variant according to this invention, either amino acid sequences or nucleic acid sequences which encode them, are sequences which are identical to a sequence of a pp32r1 polypeptide, but which differ in at least one amino acid or nucleotide residue from the sequences of human pp32 (Genbank Locus HSU73477), murine pp32 (Genbank Locus MMU73478), human cerebellar leucine rich acidic nuclear protein (LANP) (Genbank Locus AF025684), murine LANP (Genbank Locus AF022957), 11PP2a or human potent heat-stable protein phosphatase 2a inhibitor (Genbank Locus HSU60823), SSP29 (Genbank Locus HSU70439), HLA-DR associated protein I (Genbank Locus HSPPHAPI, Accession No. X75090), PHAPI2a (EMBL Locus HSPHAPI2A, Genbank Accession No. Y07569), PHAPI2b (EMBL Locus HSPHAPI2B, Genbank Accession No. Y07570), and April (EMBL Locus HSAPRIL), and preferably, are not found elsewhere in the human genome. (A list of these sequences is provided in Table 3A.) Similarly, an epitope is "unique" to pp32r1 polypeptides if it is found on pp32r1 polypeptides but not found on any members of the set of proteins listed above. Analogs of pp32r2 and unique pp32r2 sequences are defined similarly. Of course, unique sequences of pp32r1 are not found in pp32r2 and vice versa.

“Variants of pp32” are homologous proteins which differ from pp32 by at least 2 amino acids. In particular, sequence comparison between pp32 and a variant will demonstrate at least one segment of 10 amino acids in which the sequence differs by at least two (2) amino acids. More typically a variant will exhibit at least two such 10 amino acid segments. Preferably, variants of pp32 in accordance with this invention will exhibit differences in functional activity from pp32. In particular, pp32r1 and pp32r2 are variants of pp32 whose activity includes stimulation of transformation in the rat fibroblast transformation assay described herein.

A composition comprising a selected component A is “substantially free” of another component B when component A makes up at least about 75% by weight of the combined weight of components A and B. Preferably, selected component A comprises at least about 90% by weight of the combined weight, most preferably at least about 99% by weight of the combined weight. In the case of a composition comprising a selected biologically active protein, which is substantially free of contaminating proteins, it is sometimes preferred that the composition having the activity of the protein of interest contain species with only a single molecular weight (i.e., a “homogeneous” composition).

As used herein, a “biological sample” refers to a sample of tissue or fluid isolated from a individual, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, and also samples of *in vivo* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components).

“Human tissue” is an aggregate of human cells which may constitute a solid mass. This term also encompasses a suspension of human cells, such as blood cells, or a human cell line.

The term “immunoglobulin molecule” encompasses whole antibodies made up of four immunoglobulin peptide chains, two heavy chains and two light chains, as well as immunoglobulin fragments. “Immunoglobulin fragments” are protein molecules related to antibodies, which are known to retain the epitopic binding specificity of the original

antibody, such as Fab, F(ab)<sub>2</sub>, Fv, etc. Two polypeptides are "immunologically cross-reactive" when both polypeptides react with the same polyclonal antiserum.

### General Methods

The practice of the present invention employs, unless otherwise indicated, conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are well known to the skilled worker and are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook. "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins, eds., 1985); "Transcription and Translation" (B.D. Hames & S.J. Higgins, eds., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1986); "Immobilized Cells and Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989).

### 15 pp32 Related Genomic DNA

Screening a human genomic library in bacteriophages with probes generated from human pp32 cDNA yielded a new sequence that contained an open reading frame encoding a protein homologous with pp32 (see Example 2; pp32 sequence, reported in Chen, et al., *Mol. Biol. Cell*, 7:2045-2056, 1996). While the pp32r1 and pp32r2 sequences (see Figures 20 2 and 5) are substantially homologous to pp32, multiple single nucleotide base changes and short deletions suggest that they are encoded by gene distinct from pp32 gene. The pp32 family also includes substantially homologous polypeptides reported by others: HLA-DR associated protein I (Vaesen, 1994), leucine-rich acidic nuclear protein (Matsuoka, 1994), and protein phosphatase 2A inhibitor (Li, 1996).

25 DNA segments or oligonucleotides having specific sequences can be synthesized chemically or isolated by one of several approaches. The basic strategies for identifying, amplifying and isolating desired DNA sequences as well as assembling them into larger DNA molecules containing the desired sequence domains in the desired order, are well known to those of ordinary skill in the art. See, e.g., Sambrook, et al., (1989); B. Perbal, 30 (1984). Preferably, DNA segments corresponding to all or a part of the cDNA or genomic

sequence of pp32r1 may be isolated individually using the polymerase chain reaction (M.A. Innis, et al., "PCR Protocols: A Guide To Methods and Applications." Academic Press, 1990). A complete sequence may be assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g.,  
5 Edge (1981) Nature 292:756; Nambair, et al. (1984) Science 223:1299; Jay, et al. (1984) J. Biol. Chem., 259:6311.

The assembled sequence can be cloned into any suitable vector or replicon and maintained there in a composition which is substantially free of vectors that do not contain the assembled sequence. This provides a reservoir of the assembled sequence, and  
10 segments or the entire sequence can be extracted from the reservoir by excising from DNA in the reservoir material with restriction enzymes or by PCR amplification. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice (see, e.g., Sambrook, et al., incorporated herein by reference). The construction of vectors containing desired DNA segments linked by  
15 appropriate DNA sequences is accomplished by techniques similar to those used to construct the segments. These vectors may be constructed to contain additional DNA segments, such as bacterial origins of replication to make shuttle vectors (for shuttling between prokaryotic hosts and mammalian hosts), etc.

Procedures for construction and expression of proteins of defined sequence are well  
20 known in the art. A DNA sequence encoding pp32r1, pp32r2, or an analog of either pp31R1 or pp32r2, can be synthesized chemically or prepared from the wild-type sequence by one of several approaches, including primer extension, linker insertion and PCR (see, e.g., Sambrook, et al.). Mutants can be prepared by these techniques having additions, deletions and substitutions in the wild-type sequence. It is preferable to test the mutants  
25 to confirm that they are the desired sequence by sequence analysis and/or the assays described below. Mutant protein for testing may be prepared by placing the coding sequence for the polypeptide in a vector under the control of a promoter, so that the DNA sequence is transcribed into RNA and translated into protein in a host cell transformed by this (expression) vector. The mutant protein may be produced by growing host cells  
30 transfected by an expression vector containing the coding sequence for the mutant under

conditions whereby the polypeptide is expressed. The selection of the appropriate growth conditions is within the skill of the art.

The assembled sequence can be cloned into any suitable vector or replicon and maintained there in a composition which is substantially free of vectors that do not contain the assembled sequence. This provides a reservoir of the assembled sequence, and segments or the entire sequence can be extracted from the reservoir by excising from DNA in the reservoir material with restriction enzymes or by PCR amplification. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice (see, e.g., Sambrook, et al., incorporated herein by reference). The construction of vectors containing desired DNA segments linked by appropriate DNA sequences is accomplished by techniques similar to those used to construct the segments. These vectors may be constructed to contain additional DNA segments, such as bacterial origins of replication to make shuttle vectors (for shuttling between prokaryotic hosts and mammalian hosts), etc.

#### **15 Producing the Recombinant Peptide**

Preferably, DNA from the selected clones should be subcloned into an expression vector, and the protein expressed by cells transformed with the vector should be tested for immunoreactivity with antibodies against the recombinant protein of this invention prepared as described below. Such subcloning is easily within the skill of the ordinary worker in the art in view of the present disclosure. The amino acid coding region of the DNA sequence of this invention may be longer or shorter than the coding region of the disclosed sequence, so long as the recombinant peptide expressed by the DNA sequence retains at least one epitope cross-reactive with antibodies which are specifically immunoreactive with pp32r1, pp32r2, or other pp32 variant as desired. The preparation of selected clones which contain DNA sequences corresponding to all or part of the sequence of pp32r1 or pp32r2 may be accomplished by those of ordinary skill in the art using conventional molecular biology techniques along with the information provided in this specification.

It is possible to purify a pp32 variant protein, such as pp32r1, which is cross-reactive with antibodies specific for pp32, from an appropriate tissue/fluid source:



however, a cross-reactive pp32 variant, or analog thereof, may also be produced by recombinant methods from a DNA sequence encoding such a protein or polypeptide. Polypeptides corresponding to the recombinant protein of this invention may be obtained by transforming cells with an expression vector containing DNA from a clone selected from an mammalian (preferably human) library as described herein. Suitable expression vector and host cell systems are well known to those of ordinary skill in the art, and are taught, for instance, in Sambrook, et al., 1989. The peptide may be obtained by growing the transformed cells in culture under conditions wherein the cloned DNA is expressed. Of course, the peptide expressed by the clone may be longer or shorter than pp32r1 or pp32r2, so long as the peptides are immunologically cross-reactive. Depending on the expression vector chosen, the peptide may be expressed as a fusion protein or a mature protein which is secreted or retained intracellularly, or as an inclusion protein. The desired polypeptides can be recovered from the culture by well-known procedures, such as centrifugation, filtration, extraction, and the like, with or without cell rupture, depending on how the peptide was expressed. The crude aqueous solution or suspension may be enriched for the desired peptide by protein purification techniques well known to those skilled in the art. Preparation of the polypeptides may include biosynthesis of a protein including extraneous sequence which may be removed by post-culture processing.

Using the nucleotide sequences disclosed herein and the polypeptides expressed from them, antibodies can be obtained which have high binding affinity for pp32r1 or pp32r2, but much lower affinity for pp32 and/or other variants of pp32. Such antibodies, whether monoclonal or purified polyclonal antibodies can be used to specifically detect pp32r1 or pp32r2. Techniques for preparing polypeptides, antibodies and nucleic acid probes for use in diagnostic assays, as well as diagnostic procedures suitable for detection of pp32 are described in U.S. Patent Nos. 5,726,018 and 5,734,022, incorporated herein by reference, and these techniques may be applied to pp32r1 or pp32r2 by substitution of the nucleic acid sequences disclosed herein. Similar substitution may be applied to other variants of pp32.

### pp32r1 Promoter Sequence

Multiple consensus sequences for binding active steroid receptors found in genomic sequences upstream from the pp32r1 coding region are consistent with hormone regulation of gene expression. The consensus sequences were associated with the both induction and repression of expression by steroid hormones. The combination of both positively and negatively acting elements suggests complex regulation of pp32r1 expression.

Possible steroid hormone regulation of pp32r1 expression is important in regard to prostate cancer. While about one-half of treated patients initially respond to androgen ablation, subsequent hormone refraction and continued aggressive tumor growth is common (Garnick, M.B., "Prostate Cancer," in Scientific American Medicine, Dale, D.C. and Federman, D.D. Eds., Scientific American Inc., New York, 1995). Many different steroid hormones regulate the growth of prostate cancer cells (Huggins, et al., "Studies on prostate cancer: I. The effect of castration, of estrogen, and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate," *Cancer Res.*, 1:293, 1941). These findings established a basis for androgen ablation therapy for the treatment of metastatic prostate cancer.

The present invention provides androgen-activated promoters based on the upstream portion of the genomic sequence in Figure 2. The promoter sequence provided by this invention is bounded at its 3' terminus by the translation start codon of a coding sequence and extends upstream (5' direction) to include at least the number of bases or elements necessary to initiate transcription at levels above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), a protein binding domain (consensus sequence) within about 100 bases upstream of the transcription initiation site generally designated the TATA box (a binding site for TATA box binding proteins and RNA polymerase), and various other protein binding domains (consensus sequences) upstream of the TATA box that modulate the basic transcriptional activity of the transcription initiation site and the TATA box. The various other protein binding domains preferably contain recognition sequences shown in Table 1 for binding (1) androgen receptors, estrogen receptors, glucocorticoid receptors, and progesterone receptors; (2) transcription factors containing the leucine zipper motif

including, but not limited to Fos, Jun, JunB, and Myc; and, (3) certain tissue specific transcription factors including, but not limited to GATA-1 and GATA-2. The various other protein binding domains upstream of the TATA box may contribute to specificity (tissue specific expression), accuracy (proper initiation), and strength (transcription frequency) of the promoter. The promoter elements may serve overlapping functions so that the promoter may function in the absence of subsets of these elements.

### Therapy

Inhibition of function of protransforming variants of pp32 by any means would be expected to be an avenue of therapy.

U.S. Patent No. 5,726,018, incorporated herein by reference, describes various therapeutic avenues which may be applied by the skilled worker based on the nucleotides and protein sequences disclosed herein. In a particular embodiment, all or a portion of the sequence of pp32r1 or pp32r2 may be supplied in the antisense orientation to block expression of the variants found in carcinomas, particularly prostate cancer. Suitable methods for preparation of antisense expression vectors and administration of antisense therapy may be found in U.S. Patent No. 5,756,676, incorporated herein by reference. Prescreening of the patient population using the diagnostic methods described herein to identify patients having tumors expressing the particular pp32 variant is preferred.

Screening for compounds having therapeutic effects in prostate cancer may also be facilitated by the present invention. Studies which may be used to screen candidate compounds are described in U.S. Patent No. 5,756,676, incorporated herein by reference, modified by the use of cell lines which express particular variants of pp32 (see, e.g., Examples below). Compounds which affect steroid dependent protein expression may also be detected according to this invention by similar screening studies using an androgen-activated promoter as provided herein operatively coupled to a DNA sequence whose expression may be detected. (Marker sequences are well known in the art, see, e.g., Sambrook, et al., and selection of an appropriate detectable expression marker is a routine matter for the skilled worker.) Screening by testing the effect of candidate compounds on recombinant cells containing an expression vector having an androgen-activated promoter operatively coupled to an expression marker, with appropriate controls, is within the skill

of the art, in view of the promoter sequences provided herein. In one aspect, this invention provides a method for screening candidate compounds for pharmacological activity by (1) culturing a cell transfected with the DNA molecule containing an androgen-activated transcriptional promoter which is operatively linked to an open reading frame comprising  
5 at least one exon of a protein coding sequence, and (2) determining expression of the open reading frame in the presence and absence of the compound. In a preferred mode the androgen activated promoter may be the portion of the sequence in Figure 2 which is upstream of the translation initiation site, or alternatively the androgen activated promoter may be the 2700 bp upstream from the translation initiation site.

#### 10 **Diagnostic Methods Based on the pp32 Gene Family**

In one aspect, this invention provides methods for detecting and distinguishing among members of the pp32 gene family. As explained herein, the presence of one or more members of the gene family may be detected using assays based on common structures among the members resulting from common or similar sequences. For example,  
15 polyclonal antibodies elicited by pp32 will cross-react with pp32r1 and pp32r2, including various alleles of these pp32 variants. Similarly, the full coding region of the pp32 cDNA will hybridize under suitable conditions with nucleic acid encoding any of the variants, as shown by the *in situ* detection of the variants in tumor sections which were subsequently shown to contain either pp32r1 or pp32r2 allelic forms (Example 1). Selection of  
20 conditions that promote the immune cross-reactivity or cross-hybridization necessary for such detection is within the skill of the art, in view of the examples provided herein. For example, by using large nucleotide probes in hybridization experiments, the effects of one or a few differences in sequence may be overcome, i.e., larger probes will bind to more dissimilar target sequences, in contrast to shorter probes for which each nucleotide makes  
25 a larger percentage contribution to the affinity, and a single nucleotide alteration will cause a greater relative reduction in hybridization efficiency. Typically probes of 50 or more nucleotides are used to find homologues to a given sequence, and the studies reported in Example 1 used the entire sequence of pp32 as a probe to find cells expressing homologous members of the gene family other than pp32. Likewise, polyclonal antisera elicited to an  
30 antigen having multiple epitopes is more likely to cross-react with a second antigen that

has a few of the same epitopes along with many different epitopes, while a monoclonal antibody or even a purified polyclonal antiserum might not bind to the second antigen.

In addition to determining the presence of one or more members of the pp32 gene family, this invention also provides methods for distinguishing among members. 5 Determining which pp32 variant may be useful, for instance, to determine whether a transformation promoting or suppressing variant is present in a tissue sample. Suitable methods for distinguishing include both immunoassay and nucleic acid binding assays. Preferred are methods which can detect a 10-fold difference in the affinity of the detecting ligand (e.g., antibody or oligonucleotide) for the target analyte. Such methods are well 10 documented for other systems, and may be adopted to distinguish between pp32 variants by routine modification of such methods in view of the guidance provided herein.

Protein level assays may rely on monoclonal or purified polyclonal antibodies of relatively greater affinity for one variant compared to another (see, e.g., Smith, et al. ("Kinetics in interactions between antibodies and haptens," Biochemistry, 15 14(7):1496-1502, 1975, which shows that the major kinetic variable governing antibody-hapten interactions is the rate of dissociation of the complex, and that the strength of antibody-hapten association is determined principally by the activation energy for dissociation), and Pontarotti, et al. ("Monoclonal antibodies to antitumor Vinca alkaloids: thermodynamics and kinetics," Molecular Immunology, 22(3):277-84, 1985, which 20 describes a set of monoclonal antibodies that bind various dimeric alkaloids and can distinguish among the alkaloid haptens due to different relative affinities of the various monoclonal antibodies for particular dimeric alkaloids), each of which is incorporated herein by reference). Suitable modifications of the conditions for immunoassays to emphasize the relative affinity of monoclonal antibodies with different affinity are also 25 discussed in U.S. Patent No. 5,759,791, incorporated herein by reference.

A number of methods are available which are capable of distinguishing between nucleic acid sequences which differ in sequence by as little as one nucleotide. For example, the ligase chain reaction has been used to detect point mutations in various genes (see, e.g., Abravaya, et al., "Detection of point mutations with a modified ligase

chain reaction (Gap-LCR).” *Nucleic Acids Research*, 23(4):675-82, 1995, or Pfeffer, et al.,  
“A ligase chain reaction targeting two adjacent nucleotides allows the differentiation of  
cowpox virus from other Orthopoxvirus species,” *Journal of Virological Methods*,  
49(3):353-60, 1994, each of which is incorporated herein by reference). Amplification of  
5 a sequence by PCR also may be used to distinguish sequences by selection of suitable  
primers, for example, short primers, preferably 10-15 matching nucleotides, where at least  
one of the primers has on the 3' end a unique base that matches one variant but not other  
variants, and using annealing conditions under which the primer having the unique base  
has at least a ten-fold difference in dissociation rate between the fully matching variants  
10 and variants which do not fully match. Similar differentiation may be achieved in other  
methods dependent on hybridization by using short probes (typically under 50bp,  
preferably 25bp or less more preferably less than 20bp or even 10-12 bp) by adjusting  
conditions in hybridization reactions to achieve at least a ten-fold difference in dissociation  
rate for the probes between the fully matching variants and variants which do not fully  
15 match. Cleavase fragment length polymorphism may also be used, and a specific example  
below provides guidance from which the skilled worker will be able to design similar  
studies by routine selection of other cleavase enzymes in view of the sequences provided  
herein.

The diagnostic methods of this invention may be used for prognostic purposes and  
20 patient differentiation as described herein. In particular, the methods of this invention  
allow differentiation between products expressed from the various sequences disclosed in  
Figure 7. Preferred methods are those that detect and/or differentiate between pp32,  
pp32r1, and/or pp32r2. Situations in which differentiation between pp32 variants will be  
of benefit will be readily apparent to the skilled clinician, in view of the present disclosure.  
25 Selection among the diagnostic methods provided by this invention of a suitable technique  
to achieve the desired benefit is a routine matter for the skilled clinician.

### **EXAMPLES**

In order to facilitate a more complete understanding of the invention, a number of  
Examples are provided below. However, the scope of the invention is not limited to

specific embodiments disclosed in these Examples, which are for purposes of illustration only.

#### Example 1. Cellular Location of pp32 Expression

pp32 mRNA can be detected by *in situ* hybridization with a pp32 probe under  
5 stringent conditions.

**In situ hybridization.** Bases 1-298 of the pp32 cDNA sequence (GenBank H5U73477) were subcloned into the Bluescript vector by standard techniques. Digoxigenin labeled anti-sense and sense RNA probes were generated using a commercially available kit (Boehringer Mannheim). Vector DNA linearized with BamHI and XhoI served as template  
10 for antisense and sense probe generation respectively. In vitro transcription was performed for 2 hours at 37° in a final volume of 20 µl which contained 1 µg of template DNA, 2 U/µl of either T3 or T7 RNA polymerase, 1 U/µl ribonuclease inhibitor, 1 mM each of ATP, CTP, GTP, 0.65 mM UTP, 0.35 mM digoxigenin-11-UTP, 40 mM Tris-HCl pH 8.0, 10 mM NaCl, 10 mM DTT, 6 mM MgCl<sub>2</sub> and 2 mM spermidine. The reaction was  
15 stopped by adding 2 µl of 0.2M EDTA, pH 8.0 and the synthesized transcripts were precipitated for 30 min at -70° with 2.2 µl of 4 M LiCl and 75 µl of pre-chilled ethanol. RNA was pelleted by centrifugation, washed with 80% ethanol, mildly dried and dissolved in 100 µl of DEPC treated water. Yields of labeled probe were determined by an enzyme linked immunoassay using a commercially available kit (Boehringer Mannheim). Non-  
20 radioactive *in situ* hybridization was performed with anti-sense and sense pp32 RNA probes generated by *in vitro* transcription (see U.S. Patent No. 5,726,018, incorporated herein by reference). Figure 1A shows that normal prostatic basal cells are positive, whereas the clear, differentiated glandular cells are negative. In contrast, prostatic adenocarcinoma, shown at left, is strikingly positive. Note that the signal is cytoplasmic  
25 since it is mRNA and not the protein that is detected in this assay.

pp32 displays a distinctive pattern of expression *in vivo* (Chen, et al.; Malek, et al., "Identification and preliminary characterization of two related proliferation-associated nuclear phosphoproteins." *Journal of Biological Chemistry*, 265:13400-13409, 1990; Walensky, et al., "A novel M(r) 32,000 nuclear phosphoprotein is selectively expressed in  
30 cells competent for self-renewal." *Cancer Research* 53:4720-4726, 1993). In normal

peripheral tissues, expression is restricted to stem-like cell populations such as crypt epithelial cells in the gut and basal epithelium in the skin; in the adult central nervous system, cerebral cortical neurons and Purkinje cells also express pp32. In normal prostate, basal cells express pp32, whereas pp32 mRNA is not detectable by *in situ* hybridization in differentiated glandular cells (Figure 1A). In contrast, strong *in situ* hybridization to pp32 probes is found in nearly all clinically significant human prostatic adenocarcinomas. 87% of human prostatic adenocarcinomas of Gleason Score 5 and above express mRNA that hybridizes strongly with probes to pp32 in contrast to only 11% of prostate cancers of Gleason Score 4 and below in a study of 55 patients.

- 10 **Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue was sectioned at 4  $\mu$ M, deparaffinized, hydrated, processed for heat-induced antigen retrieval at 95° in 0.01 M citrate buffer, pH 6.0, for 20 min (Cattoretti, et al., "Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections," *Journal of Pathology* 171:83-98, 1993), then incubated overnight at room temperature with a 1/20 dilution of anti-pp32 antibody.
- 15 Following washing, the slide was sequentially developed with biotinylated swine-anti-rabbit IgG at 1/100 (Dako), strepavidin peroxidase (Dako), and diaminobenzidine. Figure 1B shows a representative high-grade human prostate cancer stained with affinity-purified rabbit polyclonal anti-pp32 antibody (Gusev, et al., "pp32 overexpression induces nuclear pleomorphism in rat prostatic carcinoma cells," *Cell Proliferation* 29:643-653, 1996).
- 20 The left-hand panel shows a representative field at 250x; the rectangle indicates the area shown in computer generated detail in the right-hand panel. Strongly hybridizing tumors show intense immunopositivity with antibodies to pp32, indicating that they express pp32 or immunologically related proteins (Figure 1A and 1B).

#### **Example 2. ESTs corresponding to pp32**

- 25 Several potential variant pp32 species have been identified in the prostate cancer expressed sequence tag libraries of the NCI's Cancer Genome Anatomy Project. Clone 588488 encodes a protein that is 96% identical to APRIL, although absent retrieval and sequencing of the full clone, it is impossible to tell whether the entire EST clone encodes a pp32 related sequence; neither is it possible to assess the biologic function of this
- 30 molecule at this time. Nevertheless, it is apparent that the sequenced portion encodes a



protein bearing great similarity to pp32. This EST does not appear in the database for normal prostate. As with the variant pp32 species recovered from prostate cancer, generation of this molecule by mutation would require a complex mechanism.

pp32-related genes are present in other organisms. The existence of a pp32 gene family in rodent would be consistent with the existence of a comparably sized family in human. A murine pp32 (GenBank U73478) has 89% amino acid identity to pp32, but less identity to pp32rl and APRIL. (The murine cerebellar leucine rich acidic nuclear protein has a single amino acid substitution relative to murine pp32.) We additionally identified a murine EST, GenBank AA066733, with closest identity to APRIL protein at 85% identity over 148 amino acids of a predicted open reading frame. Several other murine EST's, AA212094 and W82526, are closely related to the pp32 family but are not significantly more related to either pp32, pp32rl, or APRIL. A human homologue of such a gene would be expected to encode a fourth member of this gene family. We identified EST's predicted to encode pp32-related proteins in *C. elegans*, schistosomes, zebrafish, and *Drosophila* (data not shown). However, these sequences may not represent the complete extent of the pp32 gene family in these organisms, and thus are not informative for the likely size of the mammalian pp32 gene family.

### **Example 3. The Structure of a Gene Encoding a Relative of the pp32 Family**

Screening a human genomic library in bacteriophages with probes generated from human pp32 cDNA yielded a new sequence that contained an open reading frame encoding a protein homologous with pp32.

#### ***Screening a Human Genomic Library in Bacteriophages for pp32 cDNA:***

A genomic library from human placenta in the Lambda Fix II vector was expressed in *E. coli* strain XL-1 Blue MRA (Stratagene #946206). Screening for bacteriophage clones containing DNA inserts homologous with pp32 cDNA followed routine procedures (Sambrook, et al.). Briefly, nitrocellulose filters that had overlain bacteriophage plaques were hybridized with P-32 labeled probes for pp32 cDNA. The probes were prepared by the random primer method (Stratagene #300385) using pp32 cDNA as a template (Chen, et al., *Molec. Biol. Cell*, 7:2045-2056, 1996.). Reactive bacteriophage plaques were plugged and the bacteriophages were eluted, reexpressed, and rescreened with pp32 cDNA

probes until pure. Bacteriophage DNA was prepared by the plate lysate method (Sambrook, et al.).

*Identifying Restriction Fragments within Bacteriophage DNA Containing Sequences Homologous with pp32 cDNA.*

5 DNA from a bacteriophage clone containing pp32 cDNA sequences was digested with HindIII. Using routine methods, the restriction fragments were separated by agarose gel electrophoresis, transferred in alkaline buffer to positively charged nylon filters, and hybridized with probes that were selective for the 5' and 3' ends of the pp32 cDNA (Sambrook, et al.). The 5' and 3' probes were prepared as described above except that the  
10 products of polymerase chain reactions (PCR) were used as templates for the labeling reactions (Saiki, et al., *Science*, 239:487-491, 1988.). One PCR product was a 249 base pair segment of pp32 cDNA containing nucleotides 32 through 279. It was the result of a reaction using a pp32cDNA template and the primers

5'-TATGCTAGCGGGTTCGGGGTTTATTG-3' and

5'-GATTCTAGATGGTAAGTTTGCGATTGAGG-3' (primer set A).

The other product was a 263 base pair segment of pp32 cDNA including nucleotides 677 through 938. It was the result of a reaction using a pp32 cDNA template and the primers

5'-GAATCTAGAAGGAGGAGGAAGGTGAAGAG-3' and

5'-CTATCTAGATTCAGGGGGCAGGATTAGAG-3' (primer set B).

20 The PCR reactions included 35 cycles of one minute denaturations at 95°C, one minute primer annealings at 50°C, and one minute extensions at 72°C (cycling program A). A 4.7 kb HindIII restriction fragment that hybridized with the 5' probe, but not with the 3' probe and a 0.9 kb HindIII fragment that hybridized with the 3' probe, but not with the 5' probe were subcloned into pBluescript (Gibco) by routine methods (Sambrook, et al.). The  
25 nucleotide sequences of both strands of purified plasmid DNA containing the inserts were determined by automated procedures (DNA Analysis Facility, Johns Hopkins University School of Medicine).

**Completion of Sequencing by Direct Sequencing of PCR Products.** Alignment  
of the sequences of the 4.7 and 0.9 kb HindIII restriction fragments with pp32 cDNA  
30 showed about 90% homologies between the 3' end of the 4.7 kb fragment and the 5' region

of pp32 cDNA and the 5' end of the 0.9 kb fragment and the 3' region of the pp32 cDNA. There was an unaligned 199 base pair gap of pp32 cDNA sequence between the ends of the restriction fragments. Primers were designed to specifically anneal to relative pp32 sequences on both sides of the sequence gap. The primer sequences were

5'-GAGGTTTATTGATTGAATTCGGCT-3' and

5'-CCCCAGTACACTTTTCCCGTCTCA-3' (primer set C).

5  
Ar 7  
Polymerase chain reactions followed cycling program A with primer set C and pure bacteriophage DNA as a template. The 943 base pair products were shown by ethidium bromide staining agarose gels, extracted from excised fragments of low melt agarose  
10 (NuSieve) electrophoresis gels, and sequenced by automated procedures as described above.

A sequence of 5,785 bases was obtained from the human placental genomic library bacteriophage clone containing segments homologous with pp32 cDNA (Figure 2). This sequence was deposited in Genbank under Accession No. U71084, Locus HSU71084. The  
15 sequence has an open reading frame extending from nucleotides 4,453 to 5,154. Analysis of the nucleotide sequence upstream of the open reading frame revealed consensus sequences for active steroid hormone receptors at over twenty positions (Table 1).

Sequence analysis of the open reading frame showed 94% sequence homology to pp32 (Figure 3). Alignment of the open reading frame sequence to pp32 cDNA revealed  
20 33 scattered, solitary base differences and clustered differences of two and seven bases. There were two internal deletions of three and nine bases. The open reading frame encoded a polypeptide containing 234 amino acid residues with 88% protein-level homology to pp32 (Figure 4). Alignment of the translated sequence to the pp32 amino acid sequence revealed 18 scattered, solitary amino acid residue differences, three  
25 differences in clusters of two residues, and one difference in a clusters of four residues. There were two internal deletions of one and three residues and a terminal deletion of eleven residues. The translated sequence contained 69 acidic residues, 26 fewer than pp32.

#### **Example 4. Chromosome Mapping of pp32r1**

The pp32r1 gene maps to chromosome 4 as determined by PCR of the NIGMS  
30 monochromosomal panel 2 (Drwinga, et al., "NIGMS human/rodent somatic cell hybrid

mapping panels 1 and 2," Genomics 16:311314, 1993) followed by sequencing of the PCR product. Interestingly, the full sequence of pp32rl including 4364 nucleotides of sequence 5' to the start ATG contained over 400 matches in a blastn search of the non-redundant GenBank database. These matches were to two short regions of about 278 and 252 base  
5 pairs (nucleotides 674-952 and 2542-2794) that represent repeats in opposite orientations. The repeats are significantly related to elements on many chromosomes.

The human pp32 gene has been mapped to chromosome 15q22.3-q23 by fluorescence in situ hybridization (Fink, et al.). A Unigene entry for pp32 (Hs. 76689; HLA-DR associated protein 1) lists 93 EST sequences corresponding to this gene. 12 of  
10 which contain a mapped sequence-tagged site (STS). These STS sites are all reported to map to chromosome 15, as are many of the pp32 EST's analyzed by electronic PCR (<http://www.ncbi.nlm.nih.gov>). APRIL protein was also mapped to chromosome 15q25 (Mencinger, et al.; GenBank Y07969).

#### **Example 5. Sequence Analysis of pp32r2**

15 A pp32-related sequence (designated pp32r2) has been identified on chromosome 12 by methods analogous to those described in Example 2 for isolation of the unique intronless pp32-related gene pp32rl, found on chromosome 4. It was initially thought that the chromosome 12 sequence, encoding a truncated protein, might represent a pseudogene; however that interpretation has been reassessed in view of the present findings. The  
20 sequence has been designated pp32r2, and is recorded in Genbank as locus AF008216; the sequence of pp32r2 is shown in Figure 5. By BESTFIT analysis (Genetics Computer Group, Inc., Wisconsin Package, version 9.1, Madison, WI, 1997), pp32r2 is 99.5% identical to FT1.11, FT2.4 and T1, showing four nucleotide differences over the 875 nucleotide overlap of the sequences; this level of variation is consistent with a  
25 polymorphism. Similarly, BESTFIT analysis shows that PP32R1 is 99.6 % identical to FT3.3 and 99.4% identical to FT2.2, displaying four and five nucleotide differences, respectively (see Figure 7 below).

#### **Example 6. Sequence Comparison of Multiple Clones**

Screening of a human placental genomic library in Lambda Fix II vector  
30 (Stratagene #946206) with P-32 labeled probes for pp32 cDNA yielded a clone of

approximately 23 kb. 4.7 kb and 0.9 kb HindIII restriction fragments of this clone hybridized with probes for pp32 cDNA. The 4.7 kb clone aligned with the 5' portion of the pp32 cDNA sequence, and the 0.9 kb fragment aligned with the 3' end. A small discontinuity of 0.2 kb was sequenced from a bridging PCR product. No introns were identified.

Cultured cells including the whole human embryonic line FSH173WE and the prostatic cancer cell lines PC-3 and I.NCaP (American Type Culture Collection) were grown under recommended tissue culture conditions. Poly A+ RNA was prepared by oligo dT adsorption (MicroFasTrack, Invitrogen) and used as a template for the generation of cDNA through reactions with reverse transcriptase and random hexamers (GeneAmp RNA PCR Kit, Perkin Elmer). The cDNA sequences encoding the open reading frame were amplified by nested PCR using primers specifically selective for the genomic sequence over pp32 sequences. The final 298 base pair products were seen by ethidium bromide staining agarose electrophoretic gels.

Using procedures similar to those described in Example 3, except without the need for nested primers in most cases, transcripts from DU-145 cells and from numerous patients were sequenced for comparison to the transcripts from the above samples. The results are shown in Table 2. A summary of the degree of identity between various transcripts is provided in Table 3.

#### **Example 7. Sequence Variation for Individual Isolates of Different Cell Lines and Tumor Tissue**

The explanation for the apparent discordant expression of pp32 in cancer is that prostate tumors do not generally express pp32, but rather express variant pp32 species that promote transformation, instead of inhibiting it.

**RT-PCR and CFLP.** Sequences were reverse-transcribed and amplified using bases 32 to 52 of HSU73477 as a forward primer and 919 to 938 of the same sequence as a reverse primer in conjunction with the Titan One-Tube RT-PCR kit (Boehringer). Reverse transcription was carried out at 50° for 45 min followed by incubation at 94° for 2 min; the subsequent PCR utilized 45 cycles of 92° for 45 sec, 55° for 45 sec, and 68° for 1 min with a final extension at 68° for 10 min in a PTC 100 thermocycler (MJ Research).

Template RNA was isolated from cell lines or frozen tumor samples using RNazol B (Tel-Test) according to the manufacturer's instructions, then digested with RNase-free DNase I (Boehringer). pCMV32 was used as a positive control without reverse transcription. The cleavage assay was performed according to the manufacturer's specifications (Life Technologies) with digestion at 55° for 10 min at 0.2 mM MnCl<sub>2</sub> and electrophoresed on a 6% denaturing polyacrylamide sequencing gel.

At the level of RTPCR, paired normal prostate and prostatic adenocarcinoma from three patients yielded amplification products (Figure 6A) ranging from 889 to 909 bp. The reaction employed consensus primers capable of amplifying the full-length coding sequence from pp32 and the two closely-related intronless genomic sequences pp32r1 and pp32r2. The sole difference noted was a diminished amplicon yield from normal tissue as compared to neoplastic. Four human prostatic adenocarcinoma cell lines, DU-145, LNCaP, PC-3, and TSUPR-1, also yielded similar products.

Figure 6A shows RT-PCR amplified DNA from human prostate and prostate cancer cell lines. Lane a is an undigested control whose band migrated substantially slower than the digestion produces; samples in all other lanes were digested with cleavage as described. The lanes show: 1 kb ladder (Life Technologies), A; pCMV32. B; DU-145. C; LNCaP. D; PC-3. E; TSUPr-1. F; a representative sample, FT-1, without reverse transcription. G; FN-1. H; FT-1. I; FN-2. J; FT-2. K; FN-3. L; FT-3. M; negative control with template omitted. FN indicates frozen benign prostate and the number indicates the patient; FT indicates frozen prostatic adenocarcinoma and the number indicates the patient. Numbers on the left-hand side of the figure indicate the size in kb of a reference 1 kb DNA ladder (Life Technologies).

Qualitative differences between normal and neoplastic tissue began to emerge when the RT-PCR products were subcloned and analyzed by cleavage fragment length polymorphism analysis (CFLP) and sequence analysis. Figure 6B shows a cleavage fragment length polymorphism analysis of cloned cDNA amplified by RT-PCR from human prostatic adenocarcinoma, adjacent normal prostate, and human prostatic adenocarcinoma cell lines using primers derived from the normal pp32 cDNA sequence. The lanes show individual RT-PCR-derived clones from the DU-145, LNCaP, PC-3 and

TSUPrl cell lines, from frozen prostate cancer (FT), and from frozen normal prostate (FN):  
a, undigested normal pp32 cDNA; b, normal pp32 cDNA; c, DU-145-1; d, DU-145-3; e,  
DU-145-5; f, LNCaP-3; g, PC3-3; h, PC3-8; i, TSUPrl. -1; j, TSUPrl-3; k, TSUPrl-6; l,  
FT1.11; m, FT1.7; n, FT2.2; o, FT2.4; p, FT3.18; q, FT3.3; r, FN3.17; s, FN2.1. LNCaP  
5 expresses normal pp32. The band shifts correspond to sequence differences. All clones  
of RT-PCR product from normal prostate tissue displayed a normal CFLP pattern that  
corresponded precisely to that obtained from cloned pp32 cDNA template (GenBank  
HSU73477, Figure 6B). Prostatic adenocarcinomas yielded four distinct CFLP patterns  
upon similar analysis, of which three were unique and one mimicked the normal pp32  
10 pattern. Examination of DU-145, PC-3, and TSUPR-1 cell lines yielded substantially  
similar results whereas LNCaP yielded only a normal pp32 CFLP pattern. Further analysis  
at the sequence level confirmed that normal prostate and LNCaP contained solely normal  
pp32 transcripts.

Transcripts obtained from prostatic adenocarcinomas and from most cell lines  
15 represented closely-related variant species of pp32, summarized in Table I. These  
transcripts varied from 92.4% to 95.9% nucleotide identity to normal pp32 cDNA  
(Genetics Computer Group, Inc., Wisconsin Package, version 9.1, Madison, WI, 1997).  
Of the sixteen variant transcripts obtained, fifteen had open reading frames encoding  
proteins ranging from 89.3% to 99.6% identity to normal pp32. The table summarizes data  
20 obtained for variant pp32 transcripts obtained from human prostatic adenocarcinoma and  
prostate cancer cell lines. Sequences falling into closely related groups are indicated by  
the group letters (A,B,C); U indicates unassigned sequences not clearly falling into a  
group. The origin of each sequence is: FT, frozen tumor followed by patient number,  
decimal point, and clone number; D, DU-145 followed by clone number (as are all cell line  
25 sequences); P, PC3; and T, TSUPrl. Nucleotide identity, gaps in the nucleotide sequence  
alignment, and protein identity were determined from BESTFIT alignments with the  
normal pp32 cDNA and protein sequences. The effect on transformation is described as:  
stimulates, more foci obtained when transfected with ras+myc than with ras+myc+vector  
control; inactive, equivalent foci obtained as with ras + myc + vector control; and  
30 suppresses, fewer foci obtained as with ras + myc + vector control.

The predicted protein sequences fell into three discrete groups: [1] truncated sequences spanning the N-terminal 131 amino acids of pp32, of which one such sequence substantially equivalent to pp32r2 was obtained identically from two of three patients and from the TSUPR-1 cell line; [2] sequences more closely homologous to a distinct pp32-related gene, pp32r1 than to pp32, and [3] heterogeneous pp32-related sequences. Tumors from two of the three patients analyzed contained no detectable normal pp32 transcripts. Two of twelve cloned transcripts from the third patient tumor were normal by CFLP pattern, with sequence confirmation of normality on one clone. Two clones from cell lines were normal by CFLP screening, but were later shown to represent variant sequences.

Figures 7A and 7B show a multiple pairwise alignment of nucleotide and predicted protein sequences for all transcripts (Smith, et al., "Identification of common molecular subsequences," *J. Mol. Biol.*, 147:195-197 1981). The figures were compiled with the GCG Pileup and Pretty programs (Smith, et al.). Differences from the consensus sequences are shown as indicated; agreement with the consensus sequence is shown as a blank. Normal human pp32 is designated hpp32. Sequences from the TSUPR1, PC3, and DU-145 cell lines are as indicated. The designation FT indicates sequence derived from a frozen human prostatic adenocarcinoma. Only the normal pp32 sequence, hpp32, was obtained from normal prostate adjacent to tumor tissue. Figure 8A shows alignment of the amplicon nucleotide sequences with pp32 and the predicted amplicon from pp32r1; Figure 8B shows alignment of the predicted protein sequences. One sequence (FT 1.11), independently obtained three times from two separate patients and the TSUPR-1 cell line, is shown only once in the diagram. The pileup and pairwise alignments illustrate several important points: [1] there is a high degree of sequence conservation at both the nucleotide and predicted amino acid levels; [2] the sequence differences are distributed throughout the length of the sequence without obvious hotspots; [3] there is no obvious clustering or segmentation of sequence differences; and [4] the variant sequences fall into the previously described groups. These points are detailed in Figures 8A and 8B.

#### **Example 8. Diagnostic method to distinguish among family members**

The three members of the pp32 family which are expressed in human prostate cancer are pp32, pp32r1 and pp32r2. Whereas pp32 suppresses in vitro transformation and



in vivo tumorigenesis in model systems. pp32r1 and pp32r2 are pro-transforming and are tumorigenic in the same systems. It is possible to determine which of the three members is expressed in a tissue sample by using a protocol similar to that described in Example 7.

- 5 **Analysis from freshly frozen human tissue and cell lines.** Total RNA is extracted from freshly frozen human tissues or human cancer cell lines and subjected to reverse transcription and polymerase chain reaction amplification with single set of primers capable of amplifying the entire coding region of the cDNA of all the three genes. A suitable set of primers is:

10 Upper: 5'GGGTTGCGGGGTTTATTG3' - This corresponds to bp32 to bp48 of the pp32 cDNA sequence (Genbank U73477)  
Lower: 5'CTCTAATCCTGCCCCCTGAA3' - This corresponds to bp919 to bp938 of the pp32 cDNA sequence (Genbank U73477)

The observed amplicon sizes with this primer set are pp32 - 907bp, pp32r1 - 889bp and  
15 pp32r2 - 900bp. The three cDNAs are distinguished from each other by restriction enzyme digestion with the following enzymes - EcoR I, Hind III and Xho I. The resultant digest is run on a 2.5% agarose gel to positively identify the three different cDNAs. The table below lists the sizes of the bands observed The bolded numbers indicate the band sizes useful for identification of the three cDNAs.

20 **Table 4A Expected band sizes upon restriction digestion of the RT-PCR product from fresh tissue and cell lines**

	Undigested	EcoR I	EcoR I/Hind III Double digest	EcoR I/Xho I Double digest
hpb32	907	21.177,709	21.177.69.640	21,177,709
pp32r1	889	21.177,691	21.19.66.198,427	21.177,691
pp32r2	900	21.879	21.244,635	21.385,494

25 **Analysis from formalin fixed and paraffin embedded tissue.** A similar approach is followed for identification of pp32, pp32r1 and pp32r2 transcripts from formalin fixed and

paraffin embedded tissues. Total RNA is extracted and subjected to reverse transcription and PCR amplification with a single set of primers capable of amplifying a stretch of 200bp from all the three cDNAs. A suitable set of primers is:

Upper primer - from bp394 to bp414 of the pp32 cDNA sequence (Genbank U73477)

Lower primer - from bp609 to bp629 of the pp32 cDNA sequence (Genbank U73477)

The three cDNAs are distinguished from each other by restriction enzyme digestion with the following enzymes - Hind III, Xho I and BseR I. The resultant digest is run on a 3% agarose gel to positively identify the three different cDNAs. The table below lists the sizes of the bands observed. The bolded numbers indicate the band sizes useful for identification of the three cDNAs.

**Table 5A Expected band sizes upon restriction digestion of the RT-PCR product from formalin fixed and paraffin embedded tissues**

	Undigested	Hind III	Xho I	BseR I
hpp32	200	200	200	<b>80,120</b>
pp32r1	200	<b>100,100</b>	200	200
pp32r2	200	200	<b>44,156</b>	80,120

**Example 9. pp32r1 Augments Oncogene-Mediated Transformation of Rat Embryo Fibroblasts.**

pp32r1 was subcloned into a eukaryotic expression vector under the CMV promoter and analyzed for its effect on ras + myc-mediated formation of transformed foci in rat embryo fibroblasts. Genomic sequences including the entire coding region for pp32r1 were amplified by PCR and subcloned into the eukaryotic TA cloning and expression vector pCR3.1 vector (Invitrogen) which contains a CMV promoter. The assay was performed as described (Chen et al. Mol Biol Cell. 7:2045-56, 1996) with each T75 flask receiving 5 micrograms of pEJ-ras, and/or 10 micrograms of pMLV-c-myc, pCMV32, pp32r1 in PCR3.1, or PCR 3.1 alone. After 14 days, transformed colonies were enumerated.

Figure 8 shows the results. The data represent the average of seven replicates from two separate experiments in duplicate and one in triplicate. The error bars indicate standard error of the mean. In contrast to pp32, which consistently suppresses focus formation induced by ras + myc and other oncogene pairs, pp32r1 caused a statistically significant stimulation of focus formation with  $p=.004$  by an unpaired t-test.

#### **Example 10. Effect of Transcripts from Various Cell Lines on Rat Fibroblast Transformation Assays**

Expression constructs prepared as described above from PC-3 and DU-145 cells were tested in the rat embryo fibroblast transformation assay described by Chen, et al., *Mol Biol Cell*, 7:2045-56, 1996, incorporated herein by reference. The results are shown in Figure 9. Transcripts from the two cell lines stimulated ras+myc induction of transformed rat embryo fibroblast foci, in contrast to normal pp32, which suppressed transformation. The figure shows the mean +/- the standard deviation, except for DU-145, which represents a single determination.

#### **Example 11. Transformation Activity of Various Isolates from Patient Tumors**

The variant transcripts isolated from prostate cancer patients differ significantly from pp32 in sequence. The isolated transcripts were found to stimulate transformation. **Transformation assay.** Rat embryo fibroblasts were transfected with the indicated constructs as described (Chen, et al.) and transformed foci enumerated. For each experiment, approximately  $1 \times 10^6$  cells were plated per T75 flask and incubated for 2 to 3 d prior to transfection to achieve approximately 40% confluency. For each flask of primary rat embryo fibroblasts, the plasmids indicated in each experiment were added in the following amounts: pEJ-ras, 5  $\mu$ g; and pMLV-c-myc, pCMV32, pCMVneo, or variant pp32 constructs in pCR3.1 (Invitrogen), 10  $\mu$ g. Plasmids were prepared in two volumes Lipofectin (2  $\mu$ l lipofectin per  $\mu$ g DNA) then gently mixed by inversion in 1.5 ml OPTIMEM in sterile 15 ml polystyrene tubes and allowed to incubate at room temperature for > 15 min. For experiments with more than one flask, mixtures of all reagents were increased in proportion to the numbers of flasks required for each transfection. Cells were washed once with OPTIMEM (Gibco-BRL), and then fed with 6 ml of OPTIMEM and 1.5 ml of the DNA/Lipofectin mix. After overnight incubation, the cells were grown in

standard media and refed with fresh media twice weekly. Foci were counted fourteen days post-transfection. Figure 10 summarizes four separate experiments. Each data point represents the results from an individual flask expressed as the percent foci obtained in the contemporaneous control of ras+myc+vector.

5        Figure 10 shows that expressed variant transcripts from prostate cancer cell lines and from human prostatic adenocarcinoma generally produce increased numbers of transformed foci when co-transfected with ras and myc, as compared to the number of foci obtained when ras and myc are transfected with blank vector. Variant pp32 transcripts from DU-145 (D3), and from three prostate cancers (FT 1.7, FT 2.2, and FT3.18) yield  
10    increased numbers of transformed foci over those produced by ras and myc alone with blank vector. This stands in marked contrast to normal pp32, which consistently suppresses transformation. These activities are also summarized in Table I.

#### **Example 12. Effect of pp32 Variants on Tumorigenesis *In Vivo***

Experiments testing the effect of transfection of NIH3T3 cells on tumorigenesis *in vivo* are consistent with *in vitro* results in rat embryo fibroblasts. NIH3T3 cells were stably  
15    transfected by lipofection with the pp32 species indicated in Table 6A carried in the pCR3.1-Uni CMV-driven mammalian expression vector (Invitrogen). The G418-resistant clones employed in these experiments were all shown by genomic PCR to carry the indicated pp32 species. For analysis of tumorigenesis,  $5 \times 10^6$  cells in 100 microliters of  
20    unsupplemented Dulbecco's modified Eagle's medium without phenol red were injected into the flanks of female athymic nude mice on an outbred background of greater than six weeks in age (Harlan). For logistical reasons, inoculations of the various groups were staggered over a seven day period. Each group of mice was euthanized precisely seven weeks after inoculation. Where a mouse had a tumor, the tumor was dissected, measured,  
25    and weighed, and Table 6A reports the average weight of tumors in mice injected with cells carrying various vectors. One tumor from each group was examined histologically. All tumors were fibrosarcomas without noteworthy inflammation present. Data obtained with NIH3T3 cells indicate that NIH3T3 cells stably transfected with the variant pp32 species P3, P8, FT1.7, FT2.2, and FT2.4 form tumors when inoculated into nude mice. In  
30    contrast, NIH3T3 cells stably transfected to express human pp32 fail to form tumors *in*

*vivo* even when further transfected with *ras*. Lines of NIH3T3 cells were also established that were stably transfected with expression constructs encoding pp32 or pp32-antisense. Basal expression of pp32 is essential for maintenance of contact inhibition and serum-dependent cell growth; antisense ablation of endogenous pp32 synthesis permitted  
5 cells to grow normally following serum withdrawal. Constitutive over-expression of pp32 potentially suppressed *ras*-mediated transformation of NIH3T3 cells *in vitro* and tumorigenesis *in vivo*. In contrast, antisense ablation of endogenous pp32 dramatically increased the number and size of *ras*-transformed foci; *in vivo*, tumors obtained from *ras*-transformed antisense pp32 cells were approximately 50-fold greater in mass than tumors  
10 obtained from *ras*-transformed control cells.

For purposes of clarity of understanding, the foregoing invention has been described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains. The foregoing description and  
15 examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in medicine, immunology, hybridoma technology, pharmacology, pathology, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

20 All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

TABLE 1

Position	Strand	Consensus Sequence	Factor
4	C	TTTCCT	PEA3
21	N	CAAGGTCA	ELP
23	N	AGGTCA	PPAR
32	C	CCCTAA	TBF1
41	N	CTTGGC	NF-1 (-like proteins)
81	N	TAAACAC	Pit-1
82	N	AAACACA	HiNF-A
113	C	CTTCCC	c-Ets-2
118	N	CTATCA	GATA-1
122	N	CAGTTG	c-Myc
212	C	AATAAATA	TFIID
213	N	ATAAATA	ETF
247	N	TATCTA	NIT2
261	C	AAGGAA	c-Ets-2
262	N	AGGAAA	PEA3
283	C	TTTTTCTTTTTC	Hb
320	C	TTATAT	GAL4
333	N	TAAAAAA	TBP
349	N	TTATACATT	TBP
363	C	AAGGAA	c-Ets-2
394	C	TTTCTATA	TBP
398	N	TATAAA	TBP
398	N	TATAAA	TFIID
411	C	CTGAATT	Pit-1
420	N	TGTCCC	GR
423	C	CCCTAA	TBF1
434	N	TTCCTT	c-Ets-2
447	C	CTTCCC	c-Ets-2
514	N	TTATCTCT	GATA-1
514	C	TTATCT	GATA-2
515	N	TATCTC	NIT2
537	N	TATGCA	EFII
553	N	AAGTCA	GCN4
608	N	TGACTA	GCN4
628	N	CCTCCCAAC	LyF-1
640	N	TGTCCT	GR
648	N	TTAAAATTCA	1-Oct
648	N	TTAAAATTCA	4-Oct

Table 1 - Continued

649	N	TAAAAT	F2F
649	N	TAAAAT	Pit-1
661	N	TAAAAAA	TBP
673	N	CTTGGC	NF-1 (-like proteins)
725	N	AGGCGG	Sp1
729	N	GGGCGG	ETF
729	N	GGGCGG	Sp1
729	C	GGGCGG	Sp1
741	N	AGGTCA	PPAR
793	N	TATAAATA	B factor
793	N	TATAAA	TBP
793	N	TATAAATA	TFIID
793	N	TATAAAT	TMF
794	N	ATAAATA	ETF
809	N	TTATCT	GATA-1
809	C	TTATCT	GATA-2
815	N	GGGTGTGG	TEF-2
826	C	CACATG	muEBP-C2
826	C	CACATG	TFE3-S
826	N	CACATG	USF
978	N	ATGTAAAACA	1-Oct
978	N	ATGTAAAACA	2-Oct
978	N	ATGTAAAACA	NF-IL-2A
1000	N	ATGTCAGA	CSBP-1
1006	N	GATTTC	H4TF-1
1034	C	TTTTCAT	Pit-1
1047	N	AAGATAAAACC	RVF
1048	C	AGATAA	GATA-1
1048	N	AGATAA	GATA-2
1049	N	GATAAA	TFIID
1083	C	GCCAAG	NF-1 (-like proteins)
1124	N	CGCCAT	UCRF-L
1163	C	GACCTG	TGT3
1307	N	CAGTCA	GCN4
1347	C	TGCATA	EFII
1373	C	AGAACA	AR
1373	N	AGAACAT	GR
1373	N	AGAACA	GR
1373	C	AGAACA	GR
1373	N	AGAACA	PR
1373	C	AGAACA	PR

Table 1 - Continued

1373	N	AGAACA	PR A
1373	C	AGAACA	PR A
1393	C	TCACCT	IRF-1
1393	C	TCACCT	IRF-2
1395	C	ACTTCCT	E1A-F
1423	N	TTATCT	GATA-1
1423	C	TTATCT	GATA-2
1424	N	TATCTA	NIT2
1452	N	TTACTC	GCN4
1471	N	TGGGTCA	c-Fos
1471	N	TGGGTCA	c-Jun
1471	N	TGGGTCA	ER
1496	N	TCTCTTA	c-Myc
1511	N	TATAAA	TBP
1511	N	TATAAA	TFIID
1549	C	TTTGAA	TFIID
1568	C	AATGTATAA	TBP
1581	C	TTTGAA	TFIID
1590	C	AGATAA	GATA-1
1590	N	AGATAA	GATA-2
1591	C	GATAATTG	Dfd
1657	C	AGGACA	GR
1670	C	ATTTTA	F2F
1670	C	ATTTTA	Pit-1
1671	C	TTTTATA	B factor
1671	C	TTTTATA	Dr1
1671	C	TTTTATA	En
1671	C	TTTTATA	TBP
1671	C	TTTTATA	TBP-1
1671	C	TTTTATA	TFIIA
1671	C	TTTTATA	TFIIB
1671	C	TTTTATA	TFIID
1671	C	TTTTATA	TFIIE
1671	C	TTTTATA	TFIIF
1671	C	TTTTATA	TRF
1672	C	TTTATA	TBP
1694	C	AATAAATA	TFIID
1695	N	ATAAATA	ETF
1733	N	AGGAAA	PEA3
1749	C	TTATAT	GAL4
1783	N	TAACTCA	AP-1



Table 1 - Continued

1829	C	TAGATA	NIT2
1857	N	CGCCAT	UCRF-L
1875	N	TTCTGGGAA	IL-6 RE-BP
1895	N	TGACTA	GCN4
1899	N	TATTTAA	TBP
1942	N	ATATAA	GAL4
1985	C	TTTATA	TBP
1985	C	TTTATA	TFIID
2010	C	AATAAATA	TFIID
2011	N	ATAAATA	ETF
2058	C	TGCATA	EFII
2095	N	CAGTCA	GCN4
2146	C	AAGGAA	c-Ets-2
2147	N	AGGAAA	PEA3
2190	N	AGGAAA	PEA3
2220	C	GGCACA	GR
2252	N	CCAATAG	gammaCAAT
2286	N	TGTGCC	GR
2292	N	ATGGGA	PTF1-beta
2314	N	TATGCA	EFII
2328	C	GGCACA	GR
2350	C	ATGATAAG	GATA-1
2351	N	TGATAAG	GATA-1
2363	N	GGGAAG	c-Ets-2
2367	N	AGCCACT	CP2
2369	C	CCACTGGGGA	AP-2
2404	N	TAAAAT	F2F
2404	N	TAAAAT	F2F
2404	N	TAAAAT	Pit-1
2409	N	TTGTCATA	77+82K protein
2409	N	TTGTCATA	VETF
2415	N	TATCTA	NIT2
2451	C	TTTATC	TFIID
2452	N	TTATCT	GATA-1
2452	C	TTATCT	GATA-2
2486	N	CTCTCTCTCTC	GAGA factor
2644	N	AGGCGG	Sp1
2658	N	ACAGCTG	GT-IIBalpha
2658	N	ACAGCTG	GT-IIBbeta
2709	C	GGCCAGGC	AP-2
2723	N	TGAACT	GR

Table 1 - Continued

2731	C	TGACCT	PPAR
2731	C	TGACCTCA	URTF
2753	N	CTTGGC	NF-1 (-like proteins)
2818	C	TGATGTCA	AP-1
2818	C	TGATGTCA	c-Fos
2818	C	TGATGTCA	c-Jun
2818	C	TGATGTCA	CREB
2845	N	GGGAAG	c-Ets-2
2858	N	AGATAG	GATA-1
2858	C	AGATAG	GATA-1
2864	C	AGTTCA	GR
2899	N	ATATAA	GAL4
2900	N	TATAAAA	B factor
2900	N	TATAAAA	Drl
2900	N	TATAAAA	En
2900	N	TATAAAA	TBP
2900	N	TATAAA	TBP
2900	N	TATAAAA	TBP-1
2900	N	TATAAAA	TFIIA
2900	N	TATAAAA	TFIIB
2900	N	TATAAAA	TFIID
2900	N	TATAAAA	TFIIE
2900	N	TATAAAA	TFIIF
2900	N	TATAAAA	TRF
2921	C	TTTGAA	TFIID
2924	C	GAAATC	H4TF-1
2930	C	CATTAG	Isl-1
2948	C	TGTACA	GR
2948	C	TGTACA	PR
2948	C	TGTACA	PR A
2964	C	ATTTGAGAA	VITF
3030	N	AGTGTCT	GR
3032	N	TGTTCT	AR
3032	N	TGTTCT	GR
3032	C	TGTTCT	GR
3032	N	TGTTCT	PR
3032	C	TGTTCT	PR
3032	N	TGTTCT	PR A
3032	C	TGTTCT	PR A
3104	C	GGATTATT	TII
3106	C	ATTATTAA	AFPI

Table 1 - Continued

3111	N	TAAAAT	F2F
3111	N	TAAAAT	Pit-1
3125	C	ATTTTA	F2F
3125	C	ATTTTA	Pit-1
3142	N	TGTGAT	GR
3169	N	GTTTTATT	HOXD10
3169	N	GTTTTATT	HOXD8
3169	N	GTTTTATT	HOXD9
3175	C	TTTGAA	TFIID
3185	N	TTGCTCA	Zta
3206	N	GATTTC	H4TF-1
3212	N	AGGAAA	PEA3
3238	C	ATTTTA	F2F
3238	C	ATTTTA	Pit-1
3256	C	TTTGAA	TFIID
3266	N	TTGCTCA	Zta
3320	C	ATTTTA	F2F
3320	C	ATTTTA	Pit-1
3358	N	ATGGGA	PTF1-beta
3360	C	GGGACA	GR
3440	C	CACTCA	GCN4
3460	C	TTTCCT	PEA3
3483	N	GACACA	GR
3491	C	TTTCCT	PEA3
3495	N	CTAATG	Isl-1
3523	C	AGAACA	AR
3523	N	AGAACA	GR
3523	C	AGAACACT	GR
3523	C	AGAACA	GR
3523	N	AGAACA	PR
3523	C	AGAACA	PR
3523	N	AGAACA	PR A
3523	C	AGAACA	PR A
3538	C	TTTATC	TFIID
3539	N	TTATCT	GATA-1
3539	C	TTATCT	GATA-2
3551	N	TGAGTG	GCN4
3569	C	TCCCAT	PTF1-beta
3594	N	TTAGGG	TBF1
3653	C	CCTGCTGAA	LyF-1
3668	N	CTCATGA	1-Oct

Table 1 - Continued

3668	N	CTCATGA	2-Oct
3668	N	CTCATGA	Oct-2B
3668	N	CTCATGA	Oct-2B
3668	N	CTCATGA	Oct-2C
3679	C	TGTGTAA	Zta
3685	C	AGAACT	GR
3712	C	TTTCCT	PEA3
3713	N	TTTCCT	c-Ets-2
3717	N	TTGCTCA	Zta
3727	C	AAAACATAAAT	ssARS-T
3749	N	TAAAAAA	TBP
3784	C	CACTCA	GCN4
3791	C	ATTTTA	F2F
3791	C	ATTTTA	Pit-1
3815	N	TATCTA	NIT2
3829	C	TAGATA	NIT2
3859	C	AGAACA	AR
3859	N	AGAACAG	GR
3859	N	AGAACA	GR
3859	C	AGAACA	GR
3859	N	AGAACA	PR
3859	C	AGAACA	PR
3859	N	AGAACA	PR A
3859	C	AGAACA	PR A
3860	N	GAACAG	LVa
3877	C	ATCACA	GR
3886	N	TGAGTCA	AP-1
3886	C	TGAGTCA	AP-1
3886	C	TGAGTCA	c-Fos
3886	C	TGAGTCA	c-Jun
3886	C	TGAGTCA	Fra1
3886	C	TGAGTCA	NF-E2
3887	C	GAGTCA	GCN4
3931	N	AGATAG	GATA-1
3931	C	AGATAG	GATA-1
3960	N	TTGGCA	NF-1/L
3965	C	ATTTTA	F2F
3965	C	ATTTTA	Pit-1
4026	N	TATTTAA	TBP
4037	N	TGTGAT	GR
4040	N	GATGCAT	Pit-1

Table 1 - Continued

4042	C	TGCATA	EFII
4079	N	TTCAAAG	SRV
4079	N	TTCAAAG	TCF-1A
4079	N	TTCAAA	TFIID
4097	N	CAGGTC	TGT3
4140	N	TGATTCA	AP-1
4140	C	TGATTCA	AP-1
4140	N	TGATTC	GCN4
4164	N	GGGAGTG	p300
4205	C	AGATAA	GATA-1
4205	N	AGATAA	GATA-2
4219	C	TTAGTCAC	AP-1
4219	C	TTAGTCA	AP-1
4219	C	TTAGTCAC	c-Fos
4219	C	TTAGTCAC	c-Jun
4219	C	TTAGTCA	c-Jun
4219	C	TTAGTCA	Jun-D
4220	C	TAGTCA	GCN4
4271	N	TGTTCT	AR
4271	N	TGTTCT	GR
4271	C	TGTTCT	GR
4271	N	TGTTCT	PR
4271	C	TGTTCT	PR
4271	N	TGTTCT	PR A
4271	C	TGTTCT	PR A
4280	C	TGACCCA	c-Fos
4280	C	TGACCCA	c-Jun
4280	C	TGACCCA	ER
4292	C	CTTATCAG	GATA-1
4292	C	CTTATCA	GATA-1
4361	N	TTCAAAG	SRV
4361	N	TTCAAAG	TCF-1A
4361	N	TTCAAA	TFIID

TABLE 2

# COMPARISON OF ALL PROTEIN SEQUENCES

	1	15	16	30	31	45	46	60	61	75	76	
TSU6	MEMGRR	IIHLELRNGT	PSDVKELVLDNSRSN	EGKLEGLTDEFEEL	FLSTINVGLT	SIANL	PKLNKLLKKLELSSNR	ASVGLLEVLAEK	CPNI.	90		
D3	MEMGRR	IIHLELRNRT	PSDVKELVLDNSRSN	EGKLEGLTDEFEEL	FLSTINVGLT	SIANL	PKLNKLLKKLELSSNR	VSGGLEVLAEK	CPNI.	90		
PG	MEMGKW	IIHLELRNRT	PSDVKELFIDNSQSN	EGKLEGLADEFEEL	LINTINIGLSS	IANL	AKLNKLLKKLELSSNR	ASVGLLEVLAEK	CPNI.	90		
FT1.11	MEMGKW	IIHLELRNRT	PSDVKELFIDNSQSN	EGKLEGLTDEFEEL	LINTINIGLTS	IANL	PKLNKLLKKLELSSNR	ASVGLLEVLAEK	CPNI.	90		
TSU1	MEMGKW	IIHLELRNRT	PSDVKELFIDNSQSN	EGKLEGLTDEFEEL	LINTINIGLTS	IANL	PKLNKLLKKLELSSNR	ASVGLLEVLAEK	CPNI.	90		
FT1.18	MEMGKW	IIHLELRNRT	PSDVKELFIDNSQSN	EGKLEGLTDEFEEL	LINTINIGLTS	IANL	PKLNKLLKKLELSSNR	ASVGLLEVLAEK	CPNI.	90		
FT2.4	MEMGRR	IIHSELRNRA	PSDVKELVLDNSRSN	EGKLEGLTDSFEEL	FLSKINGGLT	SIANL	PKL-KLRKLEL---K	VSGGLEVLAEK	CPNI.	86		
FT2.2	MEMGRR	IIHSELRNRA	PSDVKELVLDNSRSN	EGKLEGLTDSFEEL	FLSKINGGLT	SIANL	PKL-KLRKLEL---R	VSGGLEVLAEK	CPNI.	86		
KG	MEMGRR	IIHLELRNRT	PSDVKELVLDNSRSN	EGKLEGLTDSFEEL	FLSKINGGLT	SIANL	PKL-KLRKLEL---R	VSGGLEVLAEK	CPNI.	86		
FT1.7	MEMGRR	IIHLELRNRT	PSDVKELVLDNSRSN	EGKLEGLTDSFEEL	FLSKINGGLT	SIANL	PKL-KLRKLEL---R	VSGGLEVLAEK	CPNI.	86		
P3	MEMGKW	IIHLELRNRT	PSDVKELFIDNSQSN	EGKLEGLTDSFEEL	LINTINIGLTS	IANL	PKLNKLLKKLELSSNR	ASVGLLEVLAEK	CPNI.	90		
L3	MEMGRR	IIHLELRNRT	PSDVKELVLDNSRSN	EGKLEGLTDSFEEL	FLSTINVOLTS	IANL	PKLNKLLKKLELSSNR	VSGGLEVLAEK	CPNI.	90		
pp32	MEMGRR	IIHLELRNRT	PSDVKELVLDNSRSN	EGKLEGLTDSFEEL	FLSTINVOLTS	IANL	PKLNKLLKKLELSSNR	VSGGLEVLAEK	CPNI.	90		
P8	MEMGRR	IIHLELRNRT	PSDVKELVLDNSRSN	EGKLEGLTDSFEEL	FLSTINVOLTS	IANL	PKLNKLLKKLELSSNR	ASVGLLEVLAEK	CPNI.	90		
TSU6	91	105	106	120	121	135	136	150	151	165	166	180
D3	IHLNLSGNKIKDLST	IEPLKKLENLES	LDL	FTCEVTNLNMY	---	---	---	---	---	---	---	131
PG	IHLNLSGNKIKDLST	IEPLKKLENLES	LDL	FTCEVTNLNMY	---	---	---	---	---	---	---	131
FT1.11	IHLNLSGNKIKDLST	IEPLKKLENLES	LDL	FTCEVTNLNMY	---	---	---	---	---	---	---	131
TSU1	IHLNLSGNKIKDLST	IEPLKKLENLES	LDL	FTCEVTNLNMY	---	---	---	---	---	---	---	131
FT1.18	IHLNLSGNKIKDLST	IEPLKKLENLES	LDL	FTCEVTNLNMY	---	---	---	---	---	---	---	131
FT2.4	IHLNLSGNKIKDLST	IEPLKKLENLES	LDL	FTCEVTNLNMY	---	---	---	---	---	---	---	131
FT2.2	THLYLSGNKIKDLST	IEPLKQLENLKS	LDL	FNCEVTNLNDYGENV	FKLLQLTYLDSCY	DIKEAPYSDIEDIVE	GLDDEEEGEHIEEYD	176				
KG	THLYLSGNKIKDLST	IEPLKQLENLKS	LDL	FNCEVTNLNDYGENV	FKLLQLTYLDSCY	DIKEAPYSDIEDIVE	GLDDEEEGEHIEEYD	176				
FT1.7	THLYLSGNKIKDLST	IEPLKQLENLKS	LDL	FNCEVTNLNDYGENV	FKLLQLTYLDSCY	DIKEAPYSDIEDIVE	GLDDEEEGEHIEEYD	176				
P3	IHLNLSGNKIKDLST	IEPLKKLENLES	LDL	FTCEVTNLNMY	REN	DUKEAPSDAEGYVE	GLDDEFEDEFEYD	180				

Ans  
N.S.

	91	105	106	120	121	135	136	150	151	165	166	
THI.NI.SGNKIKDLST	IEPLK.KLENI.KSLDL	FNCEVTNI.NDYRENV	FKLLI.PQLTYI.LDGVDYR	DUKEAPDSDAEGYVE	GLDDEEDEDDEEYD	180						
THI.NI.SGNKIKDLST	IEPLK.KLENI.KSLDL	FNCEVTNI.NDYRENV	FKLLI.PQLTYI.LDGVDYR	DUKEAPDSDAEGYVE	GLDDEEDEDDEEYD	180						
THI.NI.SGNKIKDLST	IEPLK.KLENI.KSLDL	FNCEVTNI.NDYRENV	FKLLI.PQLTYI.LDGVDYR	DUKEAPDSDAEGYVE	GLDDEEDEDDEEYD	180						
THI.NI.SGNKIKDLST	IEPLK.KLENI.KSLDL	FNCEVTNI.NDYRENV	FKLLI.PQLTYI.LDGVDYR	DUKEAPDSDAEGYVE	GLDDEEDEDDEEYD	180						

181	195 196	210 211	225 226	240 241
TSU6	-----	-----	-----	131
D3	-----	-----	-----	131
PG	-----	-----	-----	131
FT1.11	-----	-----	-----	131
TSU1	-----	-----	-----	131
FT3.18	-----	-----	-----	131
FT2.4	-----	-----	-----	234
FT2.2	-----	-----	-----	234
KG	-----	-----	-----	245
FT1.7	-----	-----	-----	249
P3	-----	-----	-----	249
L3	-----	-----	-----	249
PP12	-----	-----	-----	249
PA	-----	-----	-----	249

TSU6 and TSU1 from TSU cell line; P3 from DU-145 cell line; P3 and P8 from PC-3 cell line; FT1, FT2 and FT3 from patient carcinoma; LE from LNCAP; KG from placenta

TABLE 3

CLONE	Comparison to pp32 Sequences		
	% Identity		% Similarity
	cDNA	Protein	Protein
D3, DU-145 cells	95	90	95
P3, PC-3	86	94	96
P8, PC-3	98	97	97
FT1.11	97	86	92
FT1.7	95	95	95
FT2.2	94	85	88
FT2.4	99	86	92
FT3.18	99	90	94



TABLE 1A

Sequence	Sequence Group	Nucleotide Identity with pp32	Gaps	Protein Identity with pp32	Effect on Oncogene-Mediated Transformation	Comment
FT 1 3	A	99.8		100	Not Tested	Identical to pp32
DI	A	99.9		100	Not tested	identical to pp32 with 2 silent nt changes
L3	A	99.9		100	Not Tested	
D3	U	95.8	0	96.9	Generally Stimulatory	Encodes truncated variant pp32
D5	U	99.6	0	99.6	Not Tested	
FT 1 2	U	92.9	1		Not tested	No ORF
P3	U	96.5	1	94.4	Slightly Stimulatory	
P8	U	98.7	0	98.0	Variable	
FT 1 1	B	92.4	2	89.3	Not Tested	All sequences identical; appears to be product of pp32r2
FT 2 4	B	92.4	2	89.3	Variable	
T1	B	92.4	2	89.3		
T6	U	94.2	1	93.9	Not Tested	Encodes truncated variant pp32
FT 3 18	U	94.7	2	89.3	Stimulatory	Encodes truncated variant pp32
FT 2 2	C	94.4	3	87.6	Stimulatory	Sequences differ by 1 nt; appears to be product of pp32r1
FT 3 3	C	94.4	3	87.6	not tested	
FT 1 7	U	95.9	2	91.4	Stimulatory	

Table 2A

Protein	Genbank Accession	Length	Human pp32	Human pp32r1	Human pp32r2	Human April	Murine pp32
Human pp32	HSL173477	249	100%	88% Identity 2 gaps; Z=77	84% Identity 0 gaps; Z=73	71% Identity 3 gaps; Z=58	89% Identity 1 gap; Z=87
Human pp32r1	AF008216	234		100% Identity	785 Identity 2 gaps; Z=65	61% Identity 5 gaps; Z=15	90% identity 3 gaps; Z=64
Human pp32r2	HSL171084	131			100% Identity	61% Identity 3 gaps; Z=52	77% Identity 1 gap; Z=80
Human April	Y07969	249				100%	71% Identity 4 gaps; Z=68
Murine pp32	U734778	247					100% Identity

Percent amino acid identity of pp32 and related proteins. Sequences were aligned using the GAP program (7). The number of gaps in the alignment is indicated as well as the Z score, a statistical measure of protein relatedness derived from 50 comparisons of randomized protein sequences.

Table 3A. pp32 Homologs

human pp32 (Genbank Locus HSU73477)  
 murine pp32 (Genbank Locus MMU73478)  
 human cerebellar leucine rich acidic nuclear protein (LANP) (Genbank Locus AF025684)  
 murine LANP (Genbank Locus AF022957)  
 murine RFC1 (Genbank Locus MUSMRFC, Accession NO. L23755)  
 I1PP2a or human potent heat-stable protein phosphatase 2a inhibitor (Genbank Locus HSU60823)  
 SSP29 (Genbank Locus HSU70439)  
 HLA-DR associated protein 1 (Genbank Locus HSPHAPI, Accession No. X75090)  
 PHAPI2a (EMBL Locus HSPHAPI2A, Genbank Accession No. Y07569)  
 PHAPI2b (EMBL Locus HSPHAPI2B, Genbank Accession No. Y07570)  
 April (EMBL Locus HSAPRIL)

Table 6A. Tumorigenicity in Nude Mice of NIH3T3 Cells Transfected with pp32 and pp32 Variants

pp32 Species	Clone	Tumors/	Average Tumor Weight
FT1.7	1	3/3	14.9 ± 2.1
	2 <sup>1</sup>	3/3	13.3 ± 3.7
FT2.2	1	3/3	10.5 ± 2.8
	2	3/3	3.8 ± 2.1
FT2.4	1	3/3 <sup>6</sup>	1.3 ± 0.9
	2	3/3	13.8 ± 3.3
D3	5 <sup>2</sup>	0/3	
	6 <sup>2</sup>	0/3	
P3	11	3/3	5.7 ± 0.5
	14 <sup>3</sup>	3/3	2.1 ± 1.2
P8	1 <sup>4</sup>	3/3	6.4 ± 5.3
	2	3/3	11.3 ± 3.9
	4 <sup>5</sup>	3/3	10.1 ± 4.8
L3 (pp32)	5 <sup>3</sup>	0/3	
	6 <sup>4</sup>	0/3	
Vector Control	2 <sup>3</sup>	0/3	
	3 <sup>1</sup>	0/3	

<sup>1</sup>FT1.7, clone 2 and Vector Control, clone 3 were tested on contralateral sides of a single group of animals.  
<sup>2</sup>D3 clone 5 was tested on the contralateral sides of a group of animals simultaneously injected with NIH3T3 cells transfected with a clone of pp32r1 (data not shown). D3 clone 6 was tested on the contralateral sides of a group of animals simultaneously injected with a second clone of NIH3T3 cells transfected with pp32r1 (data not show).  
<sup>3</sup>P3, clone 14 and Vector Control, clone 2 were tested on contralateral sides of a single group of animals.  
<sup>4</sup>P8, clone 1 and pp32, clone 6 were tested on contralateral sides of a single group of animals.  
<sup>5</sup>P8, clone 4 and pp32, clone 5 were tested on contralateral sides of a single group of animals.  
<sup>6</sup>One tumor in this group, weighing 0.5 gm, was detected only upon post mortem dissection.